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(54) Tumour necrosis factor - alpha binding protein

(57) A protein or polypeptide which has the amino acid sequence of the extracellular domain of human TNF-α receptor, and in particular polypeptide having the amino acid sequence (I):
L V P H L G D R E K R D S V C P Q G K Y I H P Q N N S I C C T K C H K G T Y L Y N D C P G Q D T D C R E C E S G S
F T A S E N H L H H C L S C S K C R K E M G Q V E I S S C T V D R D T V C G C R K N Q Y R H Y W S E N L F Q C F N
C S L C L N G T V H L S C Q E K Q N T V C T C H A G F F L R E N E C V S C S N C K K S L E C T K L C L P Q I, or a derivative thereof to which human TNFα is capable of binding and whose amino acid sequence has a degree of homology of 90% or more with the sequence (I). The above amino acid sequence may be modified by removal of the first 11 aminoterminal residues and by extension at the carboxyl end with E N V K G T E D S G T T. The DNA sequence for polypeptide (I) is also given.

The protein is useful in the treatment of rheumatoid arthritis.

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At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

Fig.1.


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  1 ACCA GTGATCTCTA TGCCCGAGTC TCAACCCTCA ACTGTCACCC CAAGGCACTT GGGACGTCCT GGACAGACCG
 75 AGTCCCGGGA AGCCCCAGCA CTGCCGCTGC CACACTGCCC TGAGCCCAAA TGGGGGAGTG AGAGGCCATA GCTGTCTGGC

 40 M   G   L   S   T   V   P   D   L   L   L   P   L   V   L   L   E   L   L   V   G   I   Y   P
156 ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC

 16 S   G   V   I   G   L   V   P   H   L   G   D   R   E   K   R V D   S   V   C   P   Q   G   K
228 TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA

  9 Y   I   H   P   Q   N   N   S   I   C   C   T   K   C   H   K   G   T   Y   L   Y   N   D   C
300 TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT

 33 P   G   P   G   Q   D   T   D   C   R   E   C   E   S   G   S   F   T   A   S   E   N   H   L
372 CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC

 57 R   H   C   L   S   C   S   K   C   R   K   E   M   G   Q   V   E   I   S   S   C   T   V   D
444 AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC

 81 R   D   T   V   C   G   C   R   K   N   Q   Y   R   H   Y   W   S   E   N   L   F   Q   C   F
516 CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC

105 N   C   S   L   C   L   N   G   T   V   H   L   S   C   Q   E   K   Q   N   T   V   C   T   C
588 AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC

129 H   A   G   F   F   L   R   E   N   E   C   V   S   C   S   N   C   K   K   S   L   E   C   T
660 CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG

153 K   L   C   L   P   Q   I   E   N   V   K   G   T   E   D   S   G   T   T   V   L   L   P   L
732 AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC ACA GTG CTG TTG CCC CTG

177 V   I   F   F   G   L   C   L   L   S   L   L   F   I   G   L   M   Y   R   Y   Q   R   W   K
804 GTC ATT TTC TTT GGT CTT TGC CTT TTA TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG

201 S   K   L   Y   S   I   V   C   G   K   S   T   P   E   K   E   G   E   L   E   G   T   T   T
876 TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT
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Fig. 1(cont.)

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225  K   P   L   A   P   N   P   S   F   S   P   T   P   G   F   T   P   T   L   G   F   S   P   V
948  AAG CCC CTG GCC CCA AAC CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC AGT CCC GTG

249  P   S   S   T   F   T   S   S   S   T   Y   T   P   G   D   C   P   N   F   A   A   P   R   R
1020 CCC AGT TCC ACC TTC ACC TCC AGC TCC ACC TAT ACC CCC GGT GAC TGT CCC AAC TTT GCG GCT CCC CGC AGA

273  E   V   A   P   P   Y   Q   G   A   D   P   I   L   A   T   A   L   A   S   D   P   I   P   N
1052 GAG GTG GCA CCA CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GCC TCC GAC CCC ATC CCC AAC

297  P   L   Q   K   W   E   D   S   A   H   K   P   Q   S   L   D   T   D   D   P   A   T   L   Y
1164 CCC CTT CAG AAG TGG GAG GAC AGT GCC CAC AAG CCA CAG AGC CTA GAC ACT GAT GAC CCC GCG ACG CTG TAC

321  A   V   V   E   N   V   P   P   L   R   T   L   E   F   V   R   R   L   G   L   S   D   H   E
1236 GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC TGG AAG GAA TTC GTG CGG CGC CTA GGG CTG AGC GAC CAC GAG

345  I   D   R   L   E   L   Q   N   G   R   C   L   R   E   A   Q   Y   S   M   L   A   T   W   R
1308 ATC GAT CGG CTG GAG CTG CAG AAC GGG CGC TGC CTG CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG

369  R   R   T   P   R   R   E   A   T   L   E   L   L   G   R   V   L   R   D   M   D   L   L   G
1380 CGG CGC ACG CCG CGG CGC GAG GCC ACG CTG GAG CTG CTG GGA CGC GTG CTC CGC GAC ATG GAC CTG CTG GGC

393  C   L   E   D   I   E   E   A   L   C   G   P   A   A   L   P   P   A   P   S   L   L   R
1452 TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC GCC GCG CTC CCG CCC GCG CCC AGT CTT CTC AGA TGA
1521 GGCTGCGCCC TGCGGGCAGC TCTAAGGACC GTCCTCGCAG ATCGCCTTCC AACCCCACTT TTTTCTGGAA AGGAGGGGTC
1601 CTGCAGGGGC AAGCAGGAGC TAGCAGCCGC CTACTTGGTG CTAACCCCTC GATGTACATA GCTTTTCTCA GCTGCCTGCG
1681 CGCCGCCGAC AGTCAGCGCT GTGCGCGCGG AGAGAGGTGC GCCGTGGGCT CAAGAGCCTG AGTGGGTGGT TTGCGAGGAT
1761 GAGGGACGCT ATGCCTCATG CCCGTTTTGG GTGTCCTCAC CAGCAAGGCT GCTCGGGGGC CCCTGGTTCG TCCCTGAGCC
1841 TTTTTCACAG TGCATAAGCA GTTTTTTTTG TTTTTGTTTT GTTTTGTTTT GTTTTTTAAA TCAATCATGT TACACTAATA
1921 GAAACTTGGC ACTCCTGTGC CCTCTGCCTG GACAAGCAC ATAGCAAGCT GAACTGTCCT AAGGCAGGGG CGAGCACGGA
2001 ACAATGGGGC CTTCAGCTGG AGCTGTGGAC TTTTGTACAT ACACTAAAAT TCTGAAGTTA AG
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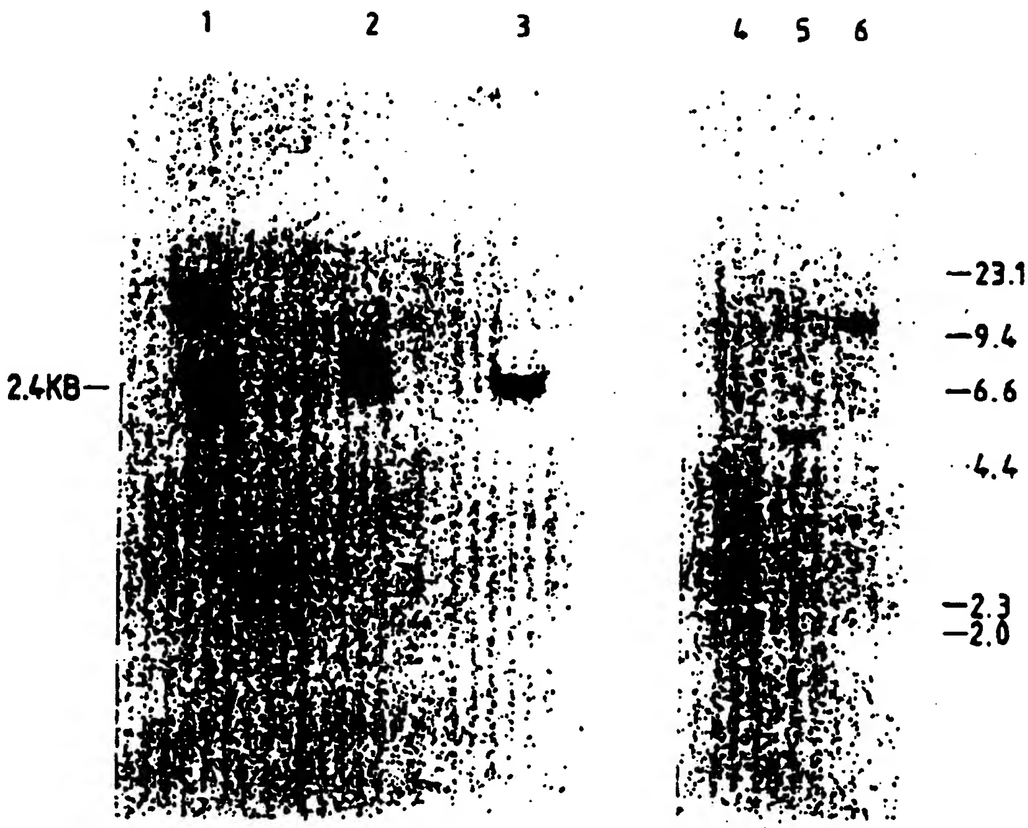
Fig. 2.



Northern blot (lanes 1-3) of 10 μg of oligo-dT selected RNA from human 293 cells (fibroblast cell line), placenta and spleen hybridised with the TNF receptor cDNA (SmaI-EcoRI fragment). The Southern blot (lanes 4-6) was hybridised with the same probe. Human genomic DNA (5 μg per lane) was digested with PstI(lane 4), Hind III (lane 5) and EcoRI (lane 6)

Fig. 3.

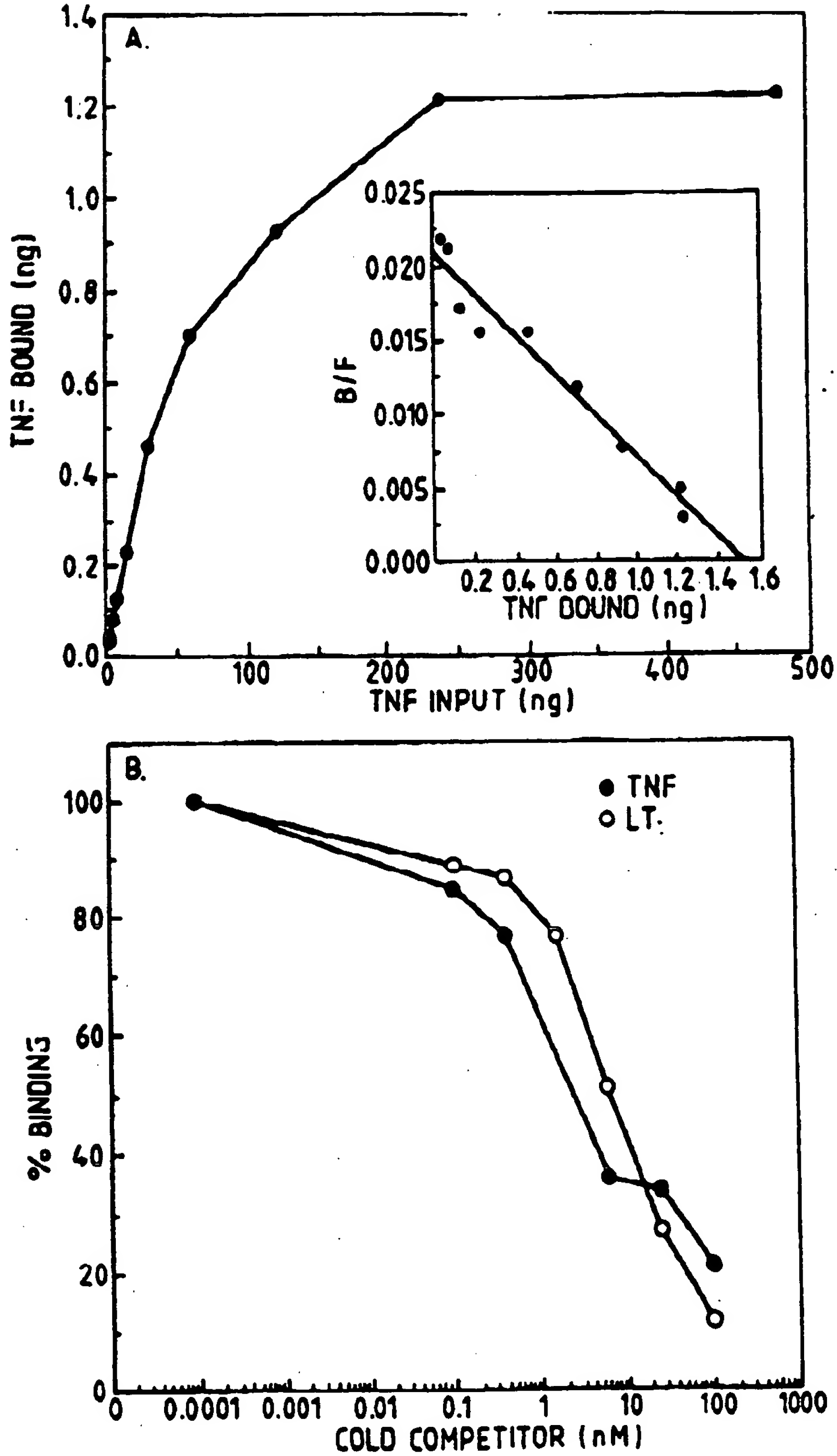
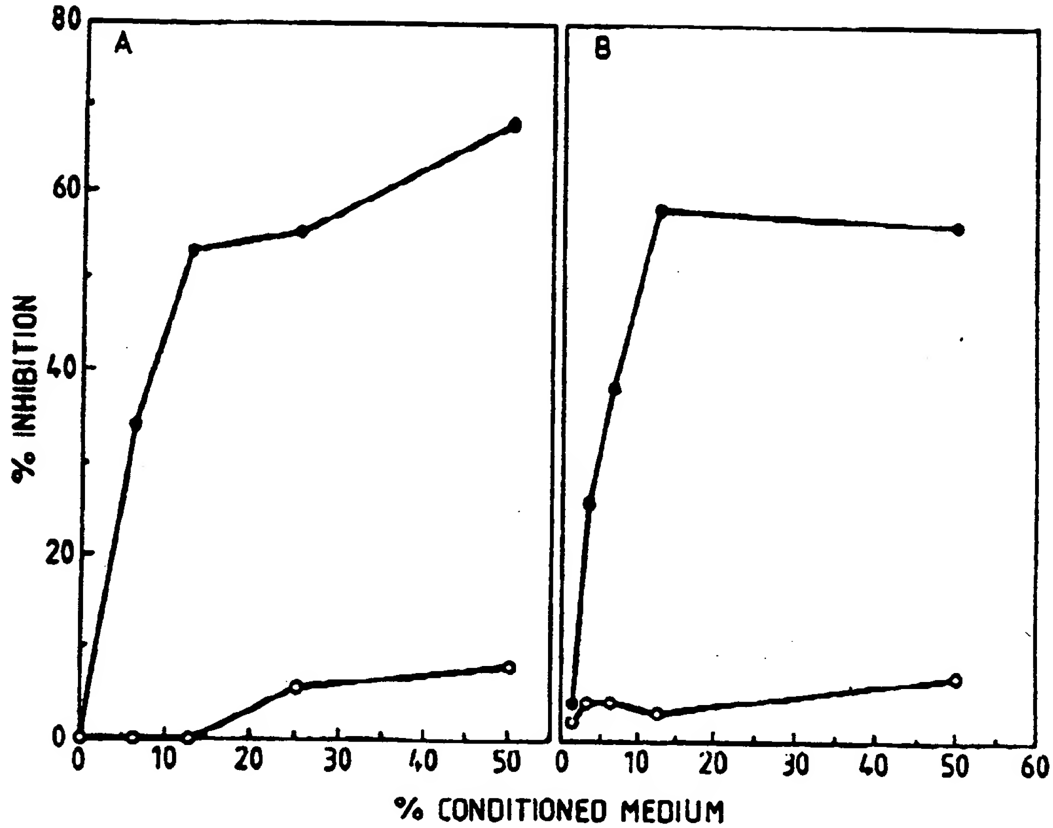

A.
TNF BOUND (ng)
TNF INPUT (ng)
B/F
TNF BOUND (ng)
B.
TNF
LT
% BINDING
COLD COMPETITOR (nM)

Fig. 4.



Effects of soluble TNF-R on TNF binding and biological activity. Panel A shows the effects of supernatants from Cos-7 cells transfected with a cDNA encoding a soluble form of the TNF receptor (pTNFRecd, closed circles) or mock transfected (open circles) on ^{125}I-TNF binding to U937 cells. Panel B shows the effects of these supernatants on TNF mediated killing of WEHI 164 (clone 13) line. Assays were performed as described in Materials and Methods

POLYPEPTIDE AND ITS USE

The present invention relates to recombinant proteins and their use.

Tumour necrosis factor-α (TNFα) is a potent cytokine which elicits a broad spectrum of biological responses. TNFα causes the cytolysis or cytostasis of many tumour cell lines *in vitro*, induces the haemorrhagic necrosis of transplanted tumours in mice, enhances the phagocytosis and cytotoxicity of polymorphonuclear neutrophils, and modulates the expression of many proteins, including lipoprotein lipase, class I antigens of the major histocompatibility complex, and cytokines such as interleukin 1 and interleukin 6. TNFα appears to be necessary for a normal immune response, but large quantities produce dramatic pathogenic effects. TNFα has been termed "cachectin" since it is the predominant factor responsible for the wasting syndrome (cachexia) associated with neoplastic disease and parasitemia. TNF is also a major contributor to toxicity in gram-negative sepsis, since antibodies against TNF can protect infected animals.

The many activities of TNFα are mediated by binding to a cell surface receptor. Radioligand binding studies have confirmed the presence of TNF receptors on a wide variety of cell types. Although these receptors are expressed in limited numbers (1,000 - 10,000 receptors/cell), they bind TNFα with high affinity ($Ka = 10^9 M^{-1}$ at 4°C). The TNF receptor has been characterised as a 65,000-80,000 dalton glycoprotein which binds both TNFα and the structurally related lymphotoxin (TNFβ). Lymphotoxin has similar, if not identical, biological activities to TNFα, presumably because both are recognized by the same receptor. Recently, several laboratories have detected heterogeneity in TNF receptor preparations, and have proposed that at least tw distinct cell surface m l cules bind TNFα. In addition, both of these recept rs appear to be released

from cells in soluble form, as TNF binding proteins of 30,000 daltons have been isolated from both urine and serum (1-3). This soluble extracellular domain retains the capacity to bind ligand with high affinity.

We have now expressed a polypeptide which corresponds to the extracellular domain of a human TNFα receptor. Further, this polypeptide is secreted as a soluble protein and is capable of binding human TNFα. The polypeptide can therefore be used in the treatment of disorders where TNFα has a significant causative role.

Accordingly, the present invention provides a polypeptide having the amino acid sequence (I):

L V P H L G D R E K R D S V C P Q G K Y
I H P Q N N S I C C T K C H K G T Y L Y
N D C P G P G Q D T D C R E C E S G S F
T A S E N H L R H C L S C S K C R K E M
C Q V E I S S C T V D R D T V C G C R K
N Q Y R H Y W S E N L F Q C F N C S L C
L N G T V H L S C Q E K Q N T V C T C H
A G F F L R E N E C V S C S N C K K S L
E C T K L C L P Q I

or a derivative thereof to which human TNFα is capable of binding and whose amino acid sequence has a degree of homology of 90% or more with the sequence (I).

The invention also provides a DNA sequence which encodes this polypeptide. The DNA sequence may be:

CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT
CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT
ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC
CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC
ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA
TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG
GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT
TAT TCC ACT CAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC
CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC

GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAC TGT GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT

The invention further provides a vector which incorporates a DNA sequence of the invention and which is capable, when provided in a transformed host, of expressing the polypeptide of the invention encoded by the DNA sequence. A host transformed with such a vector forms part of the present invention too.

In the accompanying drawings:

Figure 1 shows the nucleotide sequence of the human TNFα cDNA and encoded amino acid sequence. The predicted signal sequence residues are numbered -40 to -1. The transmembrane domain is boxed and potential N-linked glycosylation sites are overlined. The sequence homologous with the designed oligonucleotide probe is found at nucleotide positions 477-533.

Figure 2 is a Northern blot (lanes 1-3) of 10μg of oligo-dT selected RNA from human 293 cells (fibroblast cell line) (lane 1), placenta (lane 2) and spleen (lane 3) hybridised with the TNF receptor cDNA (SmaI-EcoRI fragment). The Southern blot (lanes 4-6) was hybridised with the same probe. Human genomic DNA (5 μg per lane) was digested with PstI (lane 4). Hind III (lane 5) and EcoRI (lane 6).

Figure 3 shows the binding characteristics of recombinant human TNF receptor expressed in COS-7 cells. The direct binding of recombinant ^{125}I-TNFα to COS-7 cells transfected with prTNFR is presented in panel A. The inset contains Scatchard analysis derived from this data. As shown in panel B, monolayers of Cos-7 cells transfected with TNF-R cDNA were incubated with 1nM ^{125}I-TNF in the presence of various concentrations of unlabelled TNF or LT.

Figure 4 sh ws the eff cts of soluble TNF-R n TNF binding and bi logical activity. Panel A shows th eff cts

of supernatants from Cos-7 cells transfected with a cDNA encoding a soluble form of the TNF receptor (pTNFRecd, closed circles) or mock transfected (open circles) on ^{125}I-TNF binding to U937 cells. Panel B shows the effects of these supernatants on TNF mediated killing of WEHI 164 (clone 13) line. Assays were performed as described in Materials and Methods.

In order to obtain a derivative of the polypeptide of the invention, the amino acid sequence shown above may be modified by one or more amino acid substitutions, insertions and/or deletions and/or by an extension at either or each end. A polypeptide composed of such a modified sequence must of course still be capable of binding human TNFα. Typically a modified polypeptide has a binding affinity for human TNFα of 10^7M^{-1} or greater, for example 10^8M^{-1} or greater. The affinity may be from 10^7 to $10^{10}M^{-1}$, for example from 10^8 to 10^9M^{-1}. When the unmodified amino acid sequence shown above is modified there is a degree of homology of 90% or more or 95% or more between the modified and unmodified sequence.

For example, one or more amino acid residues of the sequence above may be substituted or deleted or one or more additional amino acid residues may be inserted; provided the physicochemical character of the original sequence is preserved, i.e. in terms of charge density, hydrophobicity/hydrophilicity, size and configuration. Candidate substitutions are, based on the one-letter code (Eur. J. Biochem. <u>138</u>, 9-37, 1984):

A for G and <u>vice versa</u>,
V by A, L or G;
K by R;
S by T and <u>vice versa</u>;
E for D and <u>vice versa</u>; and
Q by N and <u>vice versa</u>.

Up to 15 residues may be deleted from the N-terminal of the polypeptide, for example up to 11 residues or up to 5 residues. As far as extensions are concerned, a short sequence of up to 50 amino acid residues may be provided at either or each terminal. The sequence may have up to 30, for example up to 20 or up to 10, amino acid residues. Up to 12 residues, for example up to 9 residues or up to 5 residues, may be added to the C-terminal of the polypeptide in the order E N V K G T E D S G T T.

Alternatively, a much longer extension may be present. Longer amino acid sequences may be fused to either or each end. A chimaeric protein may therefore be provided in which the or each extension is a heterologous amino acid sequence, i.e. a sequence not naturally linked to the amino acid sequence above. Such a chimaeric protein may therefore combine the ability to bind specifically to human TNFα with another functionality.

The polypeptides are prepared by recombinant DNA technology. The preparation of the polypeptides therefore depends upon the provision of a DNA sequence encoding the polypeptide. DNA comprising the nucleotide sequence shown above may be obtained by probing a human placenta cDNA library, for example a λgt11 library. Such a library is available from Clontech. A suitable probe is:

AAG GAG ATG GGC CAG GTT GAG ATC TCT TCT ACT GTT GAC AAT GAC ACT GTG TGT GGC

A modified form of the nucleotide sequence shown above, a polypeptide having the amino acid sequence shown above or a derivative polypeptide, may be obtained by use of any appropriate technique, including restriction with an endonuclease, insertion of linkers, use of an exonuclease and/or a polymerase and site-directed mutagenesis. Whether a m dified DNA sequenc encodes a p lypeptide of the invention can be r adily ascertained. The polyp ptide encoded by the sequence can be expressed in a suitabl host

and tested for its ability to bind specifically human TNFα.

For expression of a polypeptide of the invention, an expression vector is constructed. An expression vector is prepared which comprises a DNA sequence encoding a polypeptide of the invention and which is capable of expressing the polypeptide when provided in a suitable host. Appropriate transcriptional and translational control elements are provided, including a promoter for the DNA sequence, a transcriptional termination site, and translational start and stop codons. The DNA sequence is provided in the correct frame such as to enable expression of the polypeptide to occur in a host compatible with the vector.

The expression vector is then provided in an appropriate host. Cells harbouring the vector are grown so as to enable expression to occur. The vector may be a plasmid or a viral vector. Any appropriate host-vector system may be employed.

The transformed host may be a prokaryotic or eukaryotic host. A bacterial or yeast host may be employed, for example E. coli or S. cerevisiae. Insect cells can alternatively be used, in which case a baculovirus expression system may be appropriate. As a further alternative, cells of a mammalian cell line, such as Chinese Hamster Ovary (CHO) cells may be transformed. A polypeptide glycosylated at one, two or three of the sites shown in Figure 1 can be obtained by suitable choice of the host cell culture.

The polypeptide of the invention can be isolated and purified. The polypeptide is soluble. It can be employed in the regulation of TNF-mediated responses by binding and sequestering the cytokine. The polypeptide can therefore be used therapeutically to treat disorders such as cachexia, s psis and aut immune diseases such as rheumatoid arthritis.

For this purpose, a polypeptide of the present invention may be formulated in a pharmaceutical composition. The pharmaceutical composition also comprises a pharmaceutically acceptable carrier or diluent.

The polypeptide of the invention may be administered to a patient by any convenient route. The choice of whether an oral route or a parenteral route, such as subcutaneous, intravenous or intramuscular administration, is adopted; of the dose; and of the frequency of administration depends upon a variety of factors. These factors include the purpose of the administration, the age and weight of the patient being treated and the condition of the patient. Typically, however, the polypeptide is administered in an amount of from 1 to 1000 μg per dose, more preferably from 10 to 100 μg per dose, for each route of administration.

The invention further provides a protein which has the amino acid sequence of the extracellular domain of a human TNFα receptor, or a derivative thereof to which human TNFα is capable of binding, for use in a method of treatment of the human or animal body by therapy; especially for use in the treatment of an autoimmune disease in which TNFα plays a significant causative role, such as rheumatoid arthritis.

The extracellular domain of a TNFα receptor, or a derivative thereof capable of binding human TNFα, may therefore be used to treat rheumatoid arthritis. The extracellular domain, or a derivative thereof, of either of the two structurally distinct human TNF receptors may be used. A suitable polypeptide has the amino acid sequence (II):

D S V C P Q G K Y I H P Q N N S I C C T
K C H K G T Y L Y N D C P C P C Q D T D
C R E C E S G S F T A S E N H L R H C L
S C S K C R K E M G Q V E I S S C T V D
R D T V C G C R K N Q Y R H Y W S E N L
F Q C F N C S L C L N G T V H L S C Q E

K Q N T V C T C H A G F F L R E N E C V
S C S N C K K S L E C T K L C L P Q I E
N V K G T E D S G T T

or a derivative thereof to which human TNFα is capable of binding.

The amino acid sequence of an extracellular domain such as sequence (II) shown above may be modified by one or more amino acid substitutions, insertions and/or deletions and/or by an extension at either or each end. A polypeptide composed of such a modified sequence must of course still be capable of binding human TNFα. Typically a modified polypeptide has a binding affinity for human TNFα of $10^7 M^{-1}$ or greater, for example $10^8 M^{-1}$ or greater. The affinity may be from 10^7 to $10^{10} M^{-1}$, for example from 10^8 to $10^9 M^{-1}$. When the unmodified amino acid sequence shown above is modified there is a degree of homology of 90% or more or 95% or more between the modified and unmodified sequence.

For example, one or more amino acid residues of the sequence above may be substituted or deleted or one or more additional amino acid residues may be inserted; provided the physicochemical character of the original sequence is preserved, i.e. in terms of charge density, hydrophobicity/hydrophilicity, size and configuration. Candidate substitutions are, based on the one-letter code (Eur. J. Biochem. 138, 9-37, 1984):

A for G and *vice versa*,
V by A, L or G;
K by R;
S by T and *vice versa*;
E for D and *vice versa*; and
Q by N and *vice versa*.

Up to 15 residues may be added to the N-terminal of the polypeptide, for example up to 11 residues or up to 5 residues. As far as xtensions are concerned, a short sequence f up to 50 amino acid residues may be provided at

either or each terminal. The sequence may have up to 30, for example up to 20 or up to 10, amino acid residues. Up to 12 residues, for example up to 9 residues or up to 5 residues, may be deleted from the C-terminal of the polypeptide.

For this purpose, a polypeptide may be formulated in a pharmaceutical composition. The pharmaceutical composition also comprises a pharmaceutically acceptable carrier or diluent. The polypeptide is typically a recombinant polypeptide in pure form.

The polypeptide may be administered to a patient by any convenient route. The choice of whether an oral route or a parenteral route, such as subcutaneous, intravenous or intramuscular administration, is adopted; of the dose; and of the frequency of administration depends upon a variety of factors. These factors include the purpose of the administration, the age and weight of the patient being treated and the condition of the patient. Typically, however, the polypeptide is administered in an amount of from 1 to 1000 μg per dose, more preferably from 10 to 100 μg per dose, for each route of administration.

The following Example illustrates the invention.

EXAMPLE

1. Materials and Methods

Reagents

Recombinant human TNFα and TNFβ were supplied as highly purified proteins derived from *E. coli*. The specific activities of these preparations were approximately 10^7 units/mg, as measured in the murine L929 cell cytotoxicity assay. The synthetic oligonucleotides were prepared by Oswel DNA Service (University of Edinburgh).

Isolation of TNF receptor cDNA clones

The sequence f a peptide fragment (E M G Q V E I S S T

V D R D T V C G) of the TNF binding protein was used to design a synthetic oligonucleotide probe (5' AAG GAG ATG GGC CAG GTT GAG ATC TCT TCT ACT GTT GAC AAT GAC ACT GTG TGT CGC-3'). The 57-mer DNA probe was labelled with ^{32}P and T4 polynucleotide kinase (New England Biolab, Beverly, MA) and used to screen a placenta cDNA library in λgt10 (4,5). Approximately 800,000 phage were transferred to nitrocellulose filters and screened at reduced stringency (6). Filters were incubated for 2 hours at 42°C in 0.05M sodium phosphate, pH 6.5, 20% formamide, 0.75 M sodium chloride, 0.075 M sodium citrate, 1% polyvinyl pyrrolidone (Sigma, St Louis, MO), 1% Ficoll, 1% bovine serum albumin (Sigma), and 50 ng/ml sonicated salmon sperm DNA (Sigma). The radiolabelled probe was then added to the filters (10^6 cpm/ml final concentration) which were hybridized for 16 hours. Filters were washed extensively in 0.06M sodium chloride, 0.006M sodium citrate, 1% SDS at 37°C and positive clones were identified by autoradiography. Ten hybridising clones were plaque purified (4) and cDNA insert size was determined by polyacrylamide gel electrophoresis of EcoRI digested phage DNA. The inserts of two cDNA clones were sequenced using the dideoxy chain termination technique (7).

Southern and Northern blot analysis

DNA was isolated from human lymphocytes by the method of Blin and Stafford (8) and used for Southern blot analysis (9). DNA was digested with restriction endonucleases (New England Biolabs), fractionated on a 1% agarose gel, and transferred to nitrocellulose. Hybridisation and washing were conducted under stringent conditions (5) using a ^{32}P-labelled preparation of a 600 bp fragment of the TNF receptor cDNA. Northern blot analysis was performed (10) on oligo-dT s lected RNA is lated from human placenta, spleen (generously provided by the Cooperative Human Tissue Network, Birmingham, AL) and a fibroblast c ll line (293

cells). Following electrophoresis on a formaldehyde 1.2% agarose gel, the RNA was transferred to nitrocellulose and hybridized with the TNF receptor DNA probe under stringent conditions.

Mammalian cell expression of the human TNF receptor and derivatives

The coding region of the majority of the human TNF receptor was isolated as an EcoRI fragment and cloned into a mammalian cell expression vector (11), resulting in plasmid prTNFR. The EcoRI fragment encodes 374 amino acids of the TNF receptor; the 81 carboxyl terminal residues of the cytoplasmic domain are therefore missing from this plasmid construction. A derivative of the TNF receptor was produced by engineering a termination codon just prior to the transmembrane domain. The polymerase chain reaction (PCR) technique (12) was used to generate a 300 bp restriction fragment containing a BgIII site at the 5' end and a HindIII site preceded by a TAG stop codon at the 3' end. The PCR primers were 5'CCTGCTCCAAATGCCGAAAG and 5'AGTTCAAGCTTTTACAGTGCCCTTAACATTCTAA. The PCR product was gel purified and cloned into the TNF receptor expression plasmid (described above) digested with BgIII and HindIII. DNA sequencing confirmed that the resulting plasmid (pTNFRecd) contained the designed DNA sequence.

The TNF receptor expression plasmids were transfected into monkey COS-7 cells using Lipofectin (Gibco BRL, Bethesda, MD) according to the manufacturer's instructions. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Analysis of recombinant TNF receptor derivatives

TNFα was radioiodinated with the Iodogen method (Pierce) acc rding to the manufacturer's directions. The specific activity of the ^{125}I-TNFα was 10-30 μCU μg. COS cells

transfected with the TNF receptor cDNA (pTNFR, 1300 bp EcoRI fragment) were incubated for 24 hours and then seeded into six well tissue culture plates (Nunc) at 4.5 x 10^8 cells per well. The cells were incubated for a further 48 hours and then receptor expression was quantitated by radioligand binding for 2 hours at 4°C. Non-specific binding of ^{125}I-TNFα was determined in the presence of a 1,000 fold molar excess of unlabelled TNFα. Binding data was analysed by the method of Scatchard (13).

The TNF receptor derivative was analysed for inhibition of ^{125}I-TNFα binding to the natural receptor on human U937 cells. Culture supernatant was harvested 72 hours after COS cells were transfected with pTNFRecd. U937 cells (2 x 10^8 cells in 200 µl) were incubated with 1nM ^{125}I-TNFα and dilutions of COS cell media for 2 hours at 4°C. Cells were then centrifuged through 20% sucrose to remove unbound TNFα. Non specific binding was determined in the presence of 1µM unlabelled TNFα.

The TNF receptor derivative was also analyzed for inhibition of TNFα cytotoxic effects _in vitro_. The cytotoxicity assay was performed as described on the TNF sensitive cell line WEHI 164 clone 13 (14). Serial dilutions of supernatants from COS cells transfected with pTNFRecd or mock transfected controls were incubated with a constant amount of TNFα (1 ng/ml) for 1 hour at 27°C before addition to the assay.

2. RESULTS

Isolation and characterization of the TNF receptor cDNA

A partial amino acid sequence of the TNF binding protein was used to design a synthetic oligonucleotide probe. The radiolabelled probe was used to screen a human placenta cDNA library in λgt10 and ten hybridizing phage were isolated. The nucleotide and deduced amino acid sequences f the longest cDNA clone are d picted in Figure 1. The

third potential ATG initiation codon occurs at position 156 of the nucleotide sequence; the first two ATG codons are closely followed by termination codons, and the third ATG is preceded by the best translation initiation consensus nucleotides (15). The cDNA encodes an open reading frame of 1365 bases which codes for a polypeptide of 455 residues. Both of the peptide sequences determined by amino acid sequencing were identified in the encoded cDNA (17 of 19 and 18 of 19 matching residues). The amino terminal end identified for the TNF binding protein corresponds to the cDNA encoded sequence beginning at residue 41. The first 35 amino acids are generally quite hydrophobic and probably represent a signal sequence. Residues 38-40 are highly charged (DREKR) and such a sequence is not typically found in secretory signal sequences (16); perhaps the natural receptor is processed by proteolysis after residue 40 which contains a dibasic cleavage site (KR). Hydropathy analysis of the protein sequence predicts a single transmembrane domain of 23 amino acids. This hydrophobic sequence divides the protein into an extracellular domain of 171 residues and a cytoplasmic domain of 221 residues. The amino acid composition determined for the TNF binding protein (2) corresponds well with the predicted composition of the extracellular domain encoded by the cDNA (results not shown). The discrepancy between the predicted receptor size (40,000 daltons) and the size determined by SDS-polyacrylamide gel electrophoresis (65,000 daltons, 17-19) is probably due to glycosylation; there are four potential N-linked glycosylation sites in the sequence, three of which are in the extracellular domain. The sequence contains a large number (16) of cysteine residues, 24 of which are in the xtrac llular domain. The arrangement of these cysteine is similar t that of s veral oth r c ll surface proteins, suggesting that th TNF receptor is structurally related to

a family of receptors.

A Northern blot analysis is presented in Figure 2. The ^{32}P-labelled cDNA hybridised to a single predominant band of oligo-dT selected RNA from human placenta or spleen. A minor larger transcript was also observed in RNA from a fibroblast cell line. The size of the hybridising species is 2400 bases, in good agreement with the size of isolated cDNA. Also shown in Figure 2 is a Southern blot of human genomic DNA hybridized with a 600 bp probe from the cDNA. In each of the three different restriction digests, only a single hybridized signal was observed. Thus the TNF receptor that we have isolated appears to be encoded by a single gene.

<u>Expression of recombinant TNF receptor sequences in mammalian cells</u>

To confirm that the cDNA shown in Figure 1 indeed encodes the TNF receptor, the cDNA was engineered for expression in mammalian cells. The cDNA contains an EcoRI site at position 1270 of Figure 1. The receptor coding sequence was isolated as a 1300 bp EcoRI-fragment (containing all but the last 81 codons of the cytoplasmic domain) and inserted into a mammalian cell expression vector containing a cytomegalovirus promoter and SV40 transcription termination sequences (11). The resulting plasmid was transfected into COS cells which were analyzed for TNF receptor expression after three days. As shown in Figure 3, the transfected cells specifically bound radioiodinated TNFα in a saturable and dose dependent fashion. The population of COS cells expressed approximately 1×10^8 receptors per cell. The measured binding affinity of recombinant receptors was $2.5 \times 10^9 M^{-1}$ at 4°C which is in close agreement with natural receptor on human cells (18,19). The binding of ^{125}I-TNFα(1 nM) t these cells could be inhibited by the addition of

unlabelled TNFα or lymphotoxin (Figure 3b). COS cells transfected with just the expression vector did not significantly bind ^{125}I-TNFα (less than 2% of the binding seen with the cDNA transfection).

The extracellular domain of the TNF receptor is naturally shed from cells (1-3). To produce a similar recombinant derivative, a stop codon preceding the transmembrane domain was engineered into the cDNA by PCR mutagenesis. The modified DNA was inserted into the expression plasmid and subsequently transfected into COS cells. After three days, the COS cell media was tested for inhibition of TNFα binding to human U937 cells. As shown in Figure 4a, the transfected cell media inhibited up to 70% of the binding of TNFα. The recombinant TNF receptor derivative was next tested for inhibition of TNFα biological activity. A sensitive bioassay for TNFα is a measurement of cytolysis of mouse WEHI 164 (clone 13) cells. The transfected cell media inhibited 60% of TNFα cytotoxicity on this cell line (Figure 4b). Media from mock transfected COS cells did not inhibit TNFα induced cytotoxicity or binding. These experiments demonstrate that the recombinant extracellular domain of the TNF receptor is capable of binding TNF and inhibiting its biological activity.

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CLAIMS

1. A polypeptide having the amino acid sequence:

L V P H L G D R E K R D S V C P Q G K Y
I H P Q N N S I C C T K C H K G T Y L Y
N D C P G P G Q D T D C R E C E S G S F
T A S E N H L R H C L S C S K C R K E M
G Q V E I S S C T V D R D T V C G C R K
N Q Y R H Y W S E N L F Q C F N C S L C
L N G T V H L S C Q E K Q N T V C T C H
A G F F L R E N E C V S C S N C K K S L
E C T K L C L P Q I

or a derivative thereof to which human TNFα is capable of binding and whose amino acid sequence has a degree of homology of 90% more with the sequence (I).

2. A DNA sequence which encodes a polypeptide as defined in claim 1.

3. A DNA sequence according to claim 2, which is:

CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT
CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT
ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC
CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC
ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA
TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG
GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT
TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC
CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC
GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT
GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG
TGC CTA CCC CAG ATT

4. A DNA sequence according to claim 3, which further comprises a 5' sequence which encodes a signal amino acid sequence.

5. A vector which incorporates a DNA sequence as claimed in any one of claims 2 to 4 and which is capable, when pr vid d in a suitable h st, of expressing the said

polypeptide.

6. A vector according to claim 5, which is a plasmid.

7. A host transformed with a vector as claimed in claim 5 or 6.

8. A host according to claim 7, which is a mammalian cell line.

9. A process for the preparation of a polypeptide as defined in claim 1, which process comprises culturing a transformed host as claimed in claim 7 or 8 under such conditions that the said polypeptide is expressed.

10. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as an active principle, a polypeptide as claimed in claim 1.

11. A protein which has the amino acid sequence of the extracellular domain of a human TNFα receptor, or a derivative thereof to which human TNFα is capable of binding, for use in a method of treatment of the human or animal body by therapy.

12. A protein according to claim 11, for use in the treatment of rheumatoid arthritis.

13. A protein according to claim 11 or 12, which is a polypeptide as defined in claim 1.

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